The Journal of Neuroscience, May 1995, 15(5): 3886–3895

Contributing Factors to Poor Functional Recovery after Delayed Nerve Repair: Prolonged Denervation

Susan Y. Fu and Tessa Gordon

Department of Pharmacology and Division of Neuroscience, University of Alberta, Edmonton, Alberta, Canada T6G 2S2

The effects of prolonged denervation, independent from those of prolonged axotomy, on the recovery of muscle function were examined in a nerve cross-anastomosis paradigm. The tibialis anterior muscle was denervated for various durations by cutting the common peroneal nerve before a freshly cut tibial nerve was cross-sutured to its distal stump. Nerve regeneration and muscle reinnervation were quantified by means of electrophysiological and histochemical methods. Progressively fewer axons reinnervated the muscle with prolonged denervation; for example, beyond 6 months the mean (±SE) motor unit number was 15 ± 4, which was far fewer than that after immediate nerve suture (137 ± 21). The poor regeneration after prolonged denervation is not due to inability of the long-term denervated muscle to accept reinnervation because each regenerated axon reinnervated three- to fivefold more muscle fibers than normal. Rather, it is due to progressive deterioration of the intramuscular nerve sheaths because the effects of prolonged denervation were simulated by forcing regenerating axons to grow outside the sheaths. Fewer regenerated axons account for reinnervation of less than 50% of the muscle fibers in each muscle and contribute to the progressive decline in muscle force. Reinnervated muscle fibers failed to fully recover from denervation atrophy: muscle fiber cross-sectional area being 1171 ± 84 μm² as compared to 2700 ± 47 μm² after immediate nerve suture. Thus, the primary cause of the poor recovery after long-term denervation is a profound reduction in the number of axons that successfully regenerate through the deteriorating intramuscular nerve sheaths. Muscle force capacity is further compromised by the incomplete recovery of muscle fibers from denervation atrophy.

[Key words: prolonged denervation, regeneration, reinnervation, motor units, intramuscular nerve sheath]

The basis for the poor functional recovery after delayed nerve repair or injuries in which nerve must regenerate over long distance to reinnervate denervated target is not well understood. The poor motor recovery is generally attributed to the inability of denervated muscle to accept reinnervation and to recover from denervation atrophy (Gutmann and Young, 1944; Gutmann, 1948; reviewed by Sunderland, 1978; Irintchev et al., 1990; Terzis and Smith, 1990). However, functional recovery may also be compromised by other factors such as the reduced ability of injured motoneurons to regenerate their axons after prolonged axotomy and the deterioration of trophic and substrate support for regenerating axons in long-term denervated distal nerve stumps.

The relative contribution of prolonged axotomy and prolonged denervation to poor functional recovery can be studied independently by means of two nerve cross-anastomosis paradigms. First, prolonged axotomy can be induced by section of a nerve (motoneuron axotomy) and later cross-suture of the proximal stump of the nerve to reinnervate a freshly denervated muscle via the distal sheath of the muscle's original nerve. Second, prolonged denervation can be induced by section of a nerve (so that the target muscle and the distal nerve stump are both denervated) and later cross-suture of a freshly cut foreign nerve to the denervated distal stump of the original nerve (Holmes and Young, 1942; Gutmann and Young, 1944). Using the first paradigm along with combined physiological and histochemical methods to quantify the number of motor axons that reinnervate a muscle (motor unit or MU number) and the number of muscle fibers each motor axon had reinnervated (the innervation ratio or MU size), we showed that the regenerative capacity of injured axons was significantly compromised by prolonged axotomy (Fu et al., 1993; Fu and Gordon, 1995). In the present study, we used the second paradigm to quantify, for the first time, the effects of prolonged denervation of muscle and distal nerve sheaths on nerve regeneration and muscle reinnervation. We have extended the elegant morphological studies of Young and colleagues (Holmes and Young, 1942; Gutmann and Young, 1944) in demonstrating that the deterioration of intramuscular nerve sheaths is the major factor that limits muscle reinnervation and recovery. We also confirmed previous findings (Gutmann, 1948; Irintchev et al., 1990) that reinnervated muscle fibers fail to recover fully from denervation atrophy after long-term denervation.

The present results have been presented in abstract form (Fu et al., 1991; Fu and Gordon, 1993a,b).

Materials and Methods

Surgical procedures

Cross-suture of posterior tibial (TIB) nerve and common peroneal (CP) nerve (nerve–nerve or N-N suture) was performed in 28 adult, female Sprague–Dawley rats under sodium pentobarbital anesthesia and aseptic

Received Aug. 1, 1994; revised Dec. 5, 1994; accepted Dec. 7, 1994.

This work was supported by the Muscular Dystrophy Association and Medical Research Council of Canada. We thank the Albert Heritage Foundation of Medical Research for supporting T.G. as a research scientist, Rick Hansen Man-in-Motion Legacy Fund for supporting S.Y.F. as a research fellow, and Dr. Keir Pearson for his helpful comments on the manuscript. This work partially fulfilled the requirements for S.Y.F.'s Ph.D. thesis, for which Dr. Irvine McQuarrie was the external examiner.

Correspondence should be addressed to Dr. Susan Y. Fu, 424 Heritage Medical Research Building, University of Alberta, Edmonton, Alberta, Canada T6G 2S2.

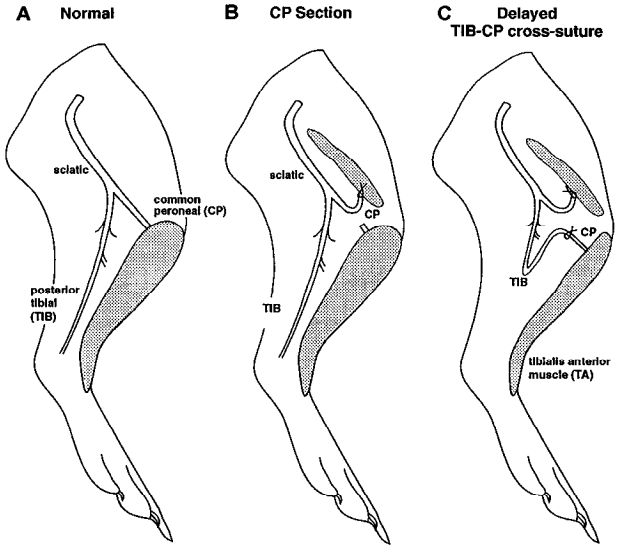

Figure 1. Diagrammatic illustration of surgical procedures. *A,* The sciatic nerve normally branches into the common peroneal (*CP*) nerve and posterior tibial (*TIB*) nerve. The CP nerve innervates muscles in the anterior compartment of the hindlimb including the tibialis anterior (*TA*) muscle (*darkened*). The TIB nerve innervates the intrinsic muscles of the foot (not shown). *B,* The CP nerve was cut and its regeneration was prevented by ligating its proximal stump and suturing it to the biceps femoris. *C,* Up to 1 year later, TIB nerve was freshly cut and its proximal stump was sutured to the previously cut distal CP stump to reinnervate the freshly or prolonged denervated TA muscle. Before the TIB-CP cross-suture, the proximal CP stump was stimulated to ensure that there was no regeneration to the TA muscle.

conditions. As shown in Figure 1, the right tibialis anterior (TA) muscle was denervated by cutting the CP nerve and ligating the nerve to prevent regeneration. At the same time, all the other muscles innervated by the CP nerve except the TA and extensor digitorum longus were removed from the anterior muscle compartment of the hindlimb. Care was taken to avoid damaging the blood supply to the TA muscle. Immediately or up to 1 year after denervation, the right TIB nerve was freshly cut and its proximal stump was sutured to the (newly or long-term denervated) distal CP nerve stump to cross-reinnervate the freshly or long-term denervated TA muscle. The proximal stump of the CP nerve was electrically stimulated to ensure that there was no CP nerve regeneration to the TA muscle.

A morphological study has shown that regenerating axons escape from the deteriorating intramuscular nerve sheaths when denervation of the muscle and distal nerve stump was prolonged prior to cross-nerve suture (Gutmann and Young, 1944). As a result, the axons grow directly on the denervated muscle surface which, as suggested, leads to poorer muscle reinnervation. If this suggestion is valid, poor reinnervation of long-term denervated muscle after cross-nerve suture should be simulated by forcing the nerve to regenerate outside the intramuscular sheaths on the denervated muscle surface. This was achieved in the present study by suturing the TIB nerve directly to the denervated TA muscle at least 8 mm away from the original nerve entry point (nerve–muscle or N-M suture). N-M suture was carried out immediately after TA muscle denervation or after the same periods of prolonged denervation as in N-N suture ($N = 33$).

At least 6 months after the N-N and N-M sutures, nerve regeneration and muscle reinnervation were evaluated in a final experiment. Rats were again anesthetized, and their blood volume was maintained by hourly intravenous injection of 5% dextrose-saline solution via a venous cannula. The trachea was cannulated for mechanical ventilation when necessary. The TA muscle was isolated by denervating all other hip, tail, and hindlimb muscles. Stimulating electrodes were inserted into the muscle beneath the sciatic nerve. Surface EMG electrodes were sewn onto the TA muscle fascia. Spinal ventral roots (L4–L6) were exposed by laminectomy. The isolated TA muscle was attached to a force transducer for muscle and MU force recordings.

Muscle and MU force recordings

Maximal muscle twitch and tetanic forces were recorded at optimal muscle length in response to suprathreshold sciatic nerve stimulation. Ventral roots were teased into small filaments, each of which contained only 3–10 motor axons innervating the TA muscle. Ventral root filaments were stimulated by gradually increasing voltage to progressively recruit single MUs as judged by all-or-none increments in twitch force and the associated unique EMG signals (Jansen and Fladby, 1990; Stein and Yang, 1990; McComas, 1991). Twitch forces of single MUs were obtained by digital subtraction.

Single MU isolation and glycogen depletion

Upon completion of MU force recordings, one MU in each muscle was isolated for further characterization into fast fatigable (FF), fast fatigue resistant (FR), fast fatigue intermediate (FI), and slow (S) on the basis of contractile speed, "sag," and fatigability (Tötösy de Zepetnek et al., 1992a). The criteria of single MU isolation were an all-or-none twitch contraction and the associated EMG response. A motor unit was selected for study only if the threshold voltage of its axon was lower than 10 V and at least 10 times less than the threshold of any other axons in the same filament. These criteria were rigidly met to keep a single MU for the subsequent 1–4 hr recordings required for adequate glycogen depletion. The isolated single MU was repetitively stimulated (starting at 1 Hz) with trains of tetanic stimuli consisting of five pulses at 50–100 Hz. These methods have been described in detail (Tötösy de Zepetnek et al., 1992a; Fu and Gordon, 1995).

Histochemistry

After the above recordings, the TA muscle was quickly removed, cut into three cross-sectional blocks, and frozen in isopentane cooled in liquid nitrogen. Muscle cross sections (10 μm) were cut and stained for Periodic Acid Schiff staining (PAS) and myosin ATPase using both acid and alkaline preincubations (Tötösy de Zepetnek et al., 1992b).

Data analysis

MU number and size. At least 30% of MUs in any reinnervated muscle were sampled to obtain a representative mean MU twitch force. The total number of MUs in each muscle was estimated by dividing the whole muscle twitch force by the mean MU twitch force.

Muscle fiber number and size. The total number of muscle fibers in each muscle was estimated by multiplying the total muscle cross-sectional area (CSA) by muscle fiber density. Muscle CSA was measured on those cross-sections that contained the maximum number of muscle fibers. The measurements of total muscle cross sectional area reflect the area of all muscle fibers and did not include the fibrotic and fatty tissues whose total area was measured and subtracted from the muscle CSA. Muscle fibers were counted in 0.63 mm^2 areas on muscle cross sections in six to nine regions that were located consistently in different muscles. Fibers within the six to nine regions represented at least 10% of all the muscle fibers in each muscle. Fiber density was measured in all the six to nine regions in order to take into account regional differences in fiber size (Pullen, 1977; Tötösy de Zepetnek et al., 1989; Parry and Wilkinson, 1991).

Two methods were used to obtain the mean muscle fiber CSA: (1) dividing the total muscle CSA by the total number of muscle fibers in each muscle and (2) directly measuring fiber CSA of 500–1000 fibers within the six to nine regions. Good agreement was found between the two methods: with mean muscle fiber CSAs being 2912 ± 63 μm^2 and 2985 ± 166 μm^2 for indirect and direct measurements, respectively, obtained from the same four rats. The indirect method was, therefore, used to acquire the mean muscle fiber CSA in all the control and reinnervated TA muscles.

Statistics

Arithmetic means were calculated and shown with standard errors (mean ± SE). One-way analysis of variance (ANOVA) was applied to examine differences in muscle force, MU number, MU force, muscle and muscle fiber CSA, and muscle fiber number between the control, cross-reinnervated muscles after immediate nerve repair and cross-reinnervated muscles after delayed nerve repair. Bonferroni tests were used to detect any differences between all the possible combinations of

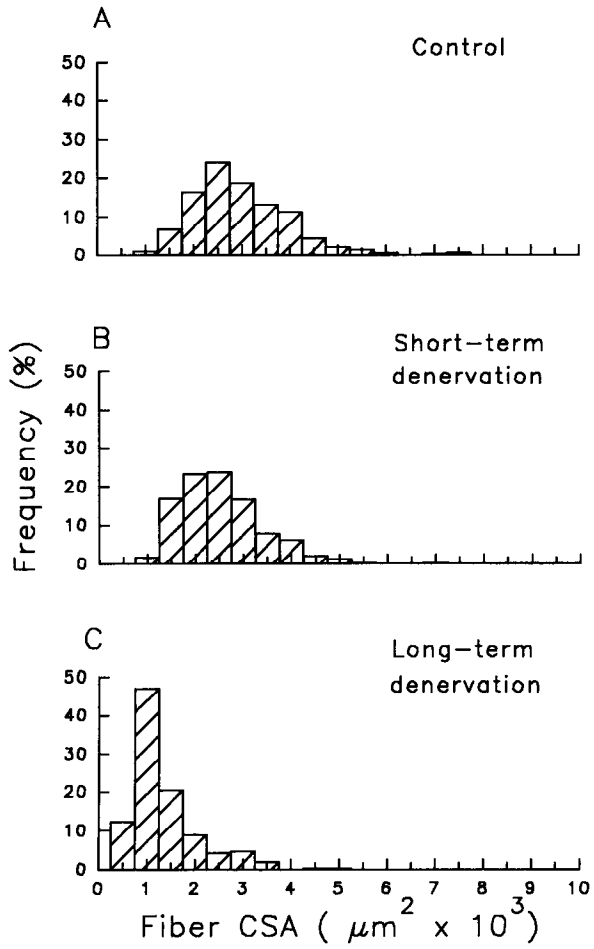

Figure 3. Frequency distribution of muscle fiber cross-sectional area in one control muscle (*A*), one reinnervated muscle after short-term denervation (*B*), and one reinnervated muscle after long-term denervation (*C*) prior to N-N suture. The mean (±SE) muscle fiber area was 2401 ± 47 μm² (*A*), 2503 ± 40 μm² (*B*), and 1031 ± 37 μm² (*C*).

paired conditions. The Kruskal–Wallis test of rank order was used to examine the differences in the distribution of muscle fiber CSA between control and cross-reinnervated muscles. Regression lines were fitted using least square analysis. For all the above statistical analyses, p values of less than 0.05 were regarded as significant.

Results

Muscle reinnervation after immediate and delayed nerve–nerve sutures

Shown in the micrographs of Figure 2, the size of a reinnervated muscle and its fibers after 1 month denervation (Fig. 2*C,D*) was comparable to the control (Fig. 2*A,B*). However, when denervation was prolonged to 6 months prior to nerve repair, the reinnervated muscle and its fibers were much smaller (Fig. 2*E,F*). As shown in the distribution of muscle fiber size in Figure 3, muscle fibers in the reinnervated muscle fully recovered from denervation atrophy when N-N suture was carried out within 45 d of denervation (Fig. 3*B*). However, reinnervated muscle fibers were much smaller when denervation was prolonged beyond 6 months prior to N-N suture (Fig. 3*C*). As summarized in Figure 4, a progressive decline in both the number (*A*) and size (*B*) of muscle fibers accounts for a smaller muscle CSA (*C*) and muscle twitch force (*D*). There was a small increase in the mean MU twitch force (*E*). The most dramatic effect of prolonged denervation was a significant decrease in the number of reinnervated MUs (*F*) and hence the number of regenerating motor axons that made functional connections with long-term denervated muscle fibers.

MU enlargement in terms of the number of muscle fibers in the MU was indicated by a small increase in MU twitch force (Fig. 4*E*) and a decrease in the size of muscle fibers (Fig. 4*B*) with prolonged muscle denervation. The innervation ratio (IR), calculated by dividing total muscle fiber number by MU number, increased significantly to partially compensate for the reduction in MU number in the reinnervated muscle (Fig. 4*F*). The average increase of threefold is as great as for intact axons that sprout in partially denervated TA muscle (Gordon et al., 1993) and regenerating axons that form enlarged MUs after immediate nerve suture. Thus, prolonged denervation does not affect the ability of motor axons to form enlarged MUs. However, MU enlargement was not sufficient to compensate for the more than 85% reduction in MU number after long-term denervation. As a result, less than 50% of the muscle fibers were reinnervated when denervation was prolonged beyond 6 months (Fig. 4*A*).

The mean maximum tetanic force (5.4 ± 0.1 N) of reinnervated muscles after immediate N-N suture was close to the contralateral control (6.0 ± 0.2 N). When the duration of denervation was prolonged for more than 6 months, the force decreased to 1.4 ± 0.26 N. This reduction was accompanied by hypertrophy of the contralateral control TA muscle (8.9 ± 0.26 N), presumably as a result of the overuse to compensate for the "foot drop" in the experimental limb.

Muscle reinnervation after immediate and delayed nerve–muscle sutures

The success of reinnervation of short-term (<45 d) denervated muscles observed after N-N suture was not seen after N-M suture (Fig. 5). Even immediate N-M suture led to a significant reduction in the number of reinnervated MUs (Fig. 5*F*) and an associated decrease in muscle CSA (Fig. 5*C*), muscle force (Fig. 5*D*), and muscle fiber number (Fig. 5*A*).

Thus, poor reinnervation of long-term denervated muscle after N-N suture was simulated by forcing axons to regenerate outside the intramuscular nerve sheaths (N-M suture). This provided evidence to support the idea that poor reinnervation of long-term denervated muscles after N-N suture can be explained by escape of regenerating axons from the deteriorating intramuscular nerve sheaths and elongation on the surface of denervated muscle fibers.

To determine whether the growth substrate of denervated muscle also deteriorates with time, denervation was prolonged prior to N-M suture. A 2–6 week delay prior to N-M suture reduced the number of reinnervated MUs to an average of 10 in comparison with 84 ± 4 for the same delay prior to N-N suture (Fig. 5*F*). Thus, the growth substrate of denervated muscle deteriorates more rapidly than that of the denervated intramuscular nerve sheaths. Possibly as a result of deterioration of the sheaths, there were similarly few reinnervated MUs under conditions of both N-N and N-M sutures after the target muscle and the intramuscular nerve sheaths had been denervated for 6 months.

The effects on muscle reinnervation of N-N and N-M sutures and of immediate and delayed nerve repair are compared in Figure 6. After immediate nerve suture (no delay in Fig. 6), regeneration (MU number, Fig. 6*A*) was significantly less successful after N-M suture than after N-N suture. The capacity of

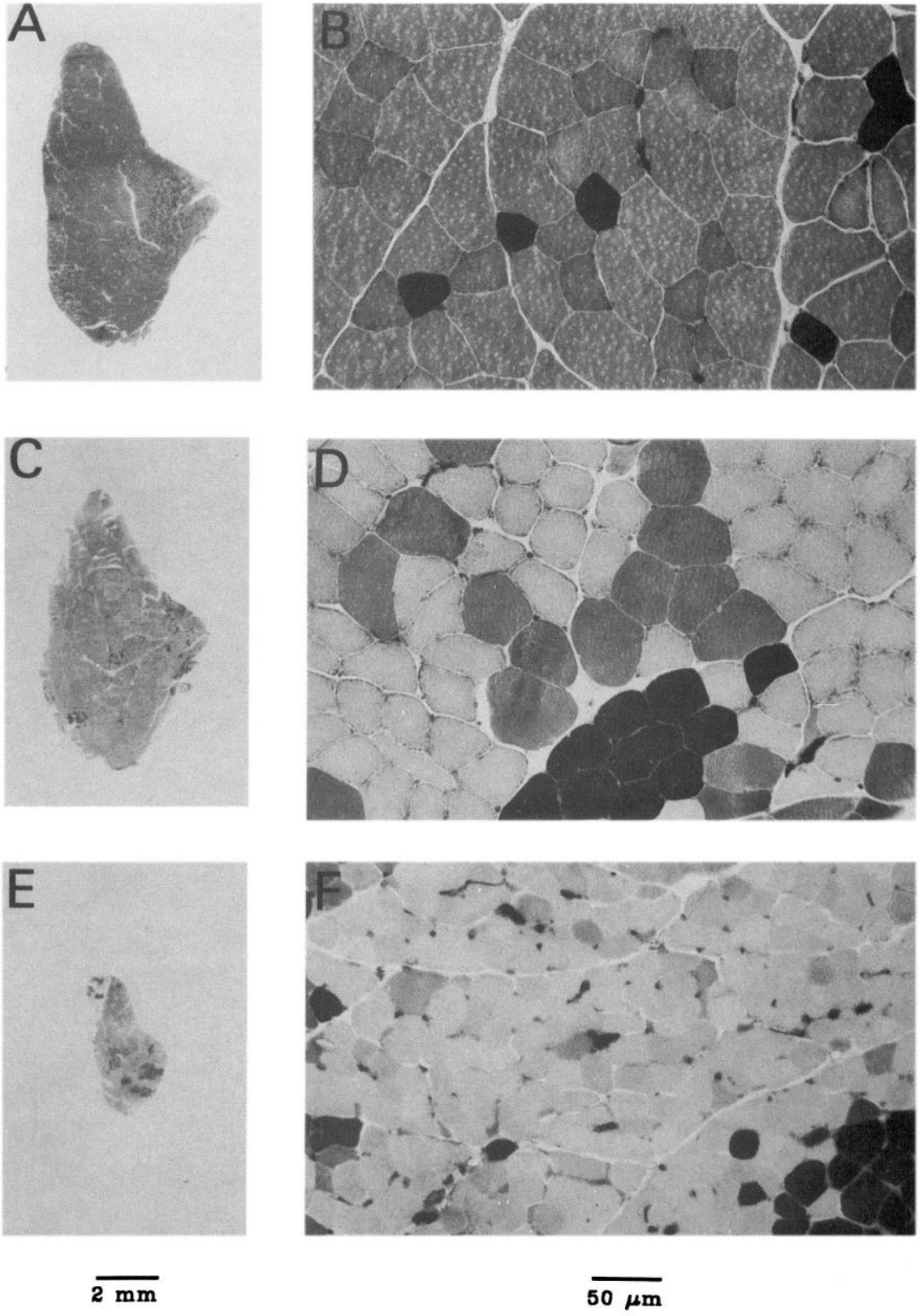

Figure 2. Low- and higher power photomicrographs of cross-sections of control (*A* and *B*), cross-reinnervated muscle after short-term (1 month) denervation (*C* and *D*), and long-term (12 month) denervation (*E* and *F*) prior to N-N suture. The size of the reinnervated muscle and its muscle fibers was similar to the control after short-term denervation prior to nerve repair but was much smaller than the control when denervation was prolonged 12 months prior to nerve repair.

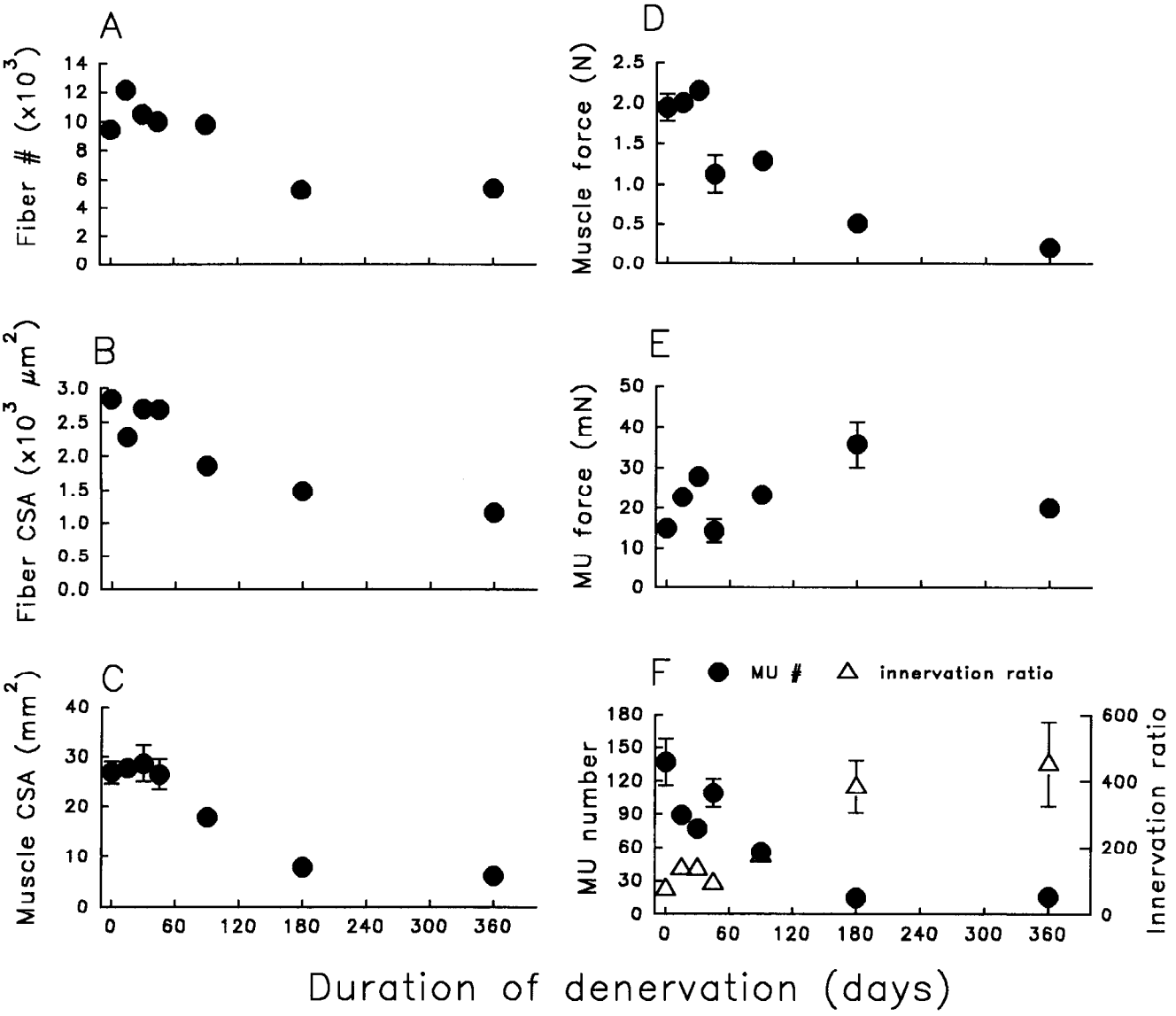

Figure 4. Effect of muscle denervation prior to TIB-CP cross-suture on mean (±SE) total muscle fiber number (*A*), muscle fiber CSA (*B*), muscle cross-sectional area (CSA) (*C*), twitch force (*D*), MU twitch force (*E*), MU number (*F, the left y-axis*), and innervation ratio (*F, the right y-axis*) in reinnervated TA muscle.

the few regenerated axons to form enlarged MUs after N-M suture (Fig. 6*B*) partially compensated for the reduction in MU number. As a result, almost as many muscle fibers were reinnervated (Fig. 6*C*) and muscle force recovered almost as well as after N-N suture (Fig. 6*E*). Denervated muscle fibers completely recovered from denervation atrophy after both N-N and N-M sutures (Fig. 6*D*).

After prolonged denervation (>6 months; prolonged delay in Fig. 6), MU number was equally reduced after N-N and N-M sutures to less than 12% of that after immediate N-N suture (Fig. 6*A*). After delayed N-N suture, the capacity of motor axons to form enlarged MUs partially compensated for the reduction in MU number (Fig. 6*B*). However, the relatively poor reinnervation of denervated muscle fibers (4901 ± 830 and 10006 ± 470 after delayed and immediate N-N sutures, respectively) combined with the failure of long-term denervated muscle fibers to fully recover from denervation atrophy (Fig. 6*D*), resulted in less than 25% muscle force of that after immediate N-N suture (Fig. 6*E*). After delayed N-M suture, IR was not increased to compensate for low MU number (Fig. 6*B*), with the result that muscle force was less than that after delayed N-N suture. Thus, long-term denervation reduces both the number of motor axons that successfully reinnervated the muscle and the capacity of motor axons to form enlarged MUs after N-M suture.

Relationship between IR and MU force after N-N and N-M sutures

One MU per muscle was glycogen-depleted to obtain direct counts of MU fibers (IR). MU fibers were generally clumped and of the same histochemical type (Fu et al., 1992). There was a higher proportion of FI units (Fig. 7) than normal (21%) as previously described in self-reinnervated muscles after immediate nerve–nerve suture (Tötösy de Zepetnek et al., 1992a).

In agreement with the data on self-reinnervated TA muscles (Tötösy de Zepetnek et al., 1992a), IR was strongly correlated with MU tetanic force in cross-reinnervated muscles and that IR

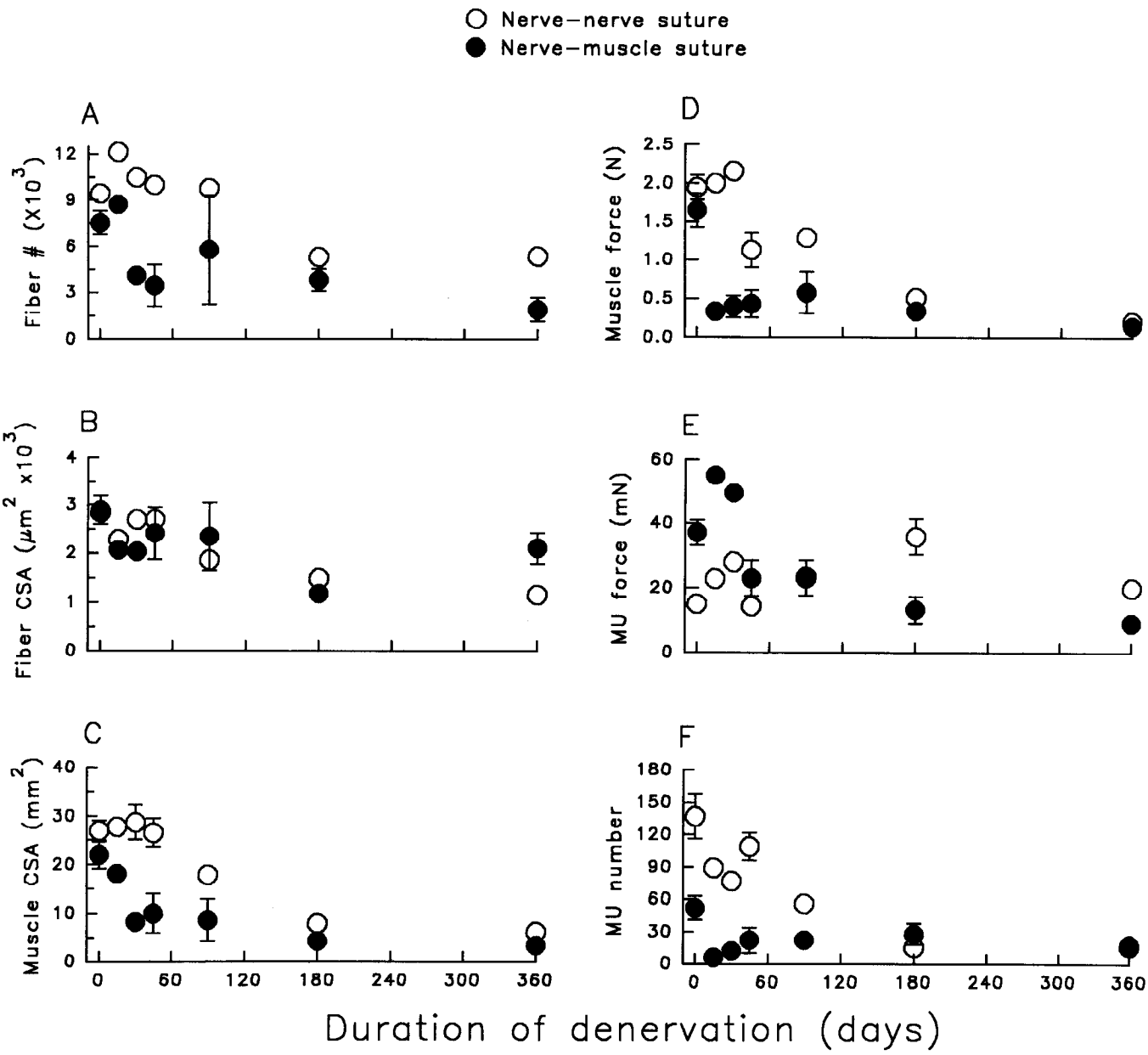

Figure 5. Effects of denervation on reinnervated muscle fiber number (*A*), muscle fiber cross-sectional area (CSA) (*B*), muscle CSA (*C*), muscle force (*D*), MU force (*E*), and MU number (*F*) after N-M suture (*filled circles*) compared with those after N-N suture (*open circles*). Data points were expressed as means ± SE.

and force increased in the order of FR < FI < FF (Fig. 7*A,C,E*). The same correlations were also found between IR and twitch force (Fig. 7*B,D,F*). Interestingly, the slopes of the regression lines were the same for N-N and N-M sutures and for short and long periods of denervation. Thus, the relationship between IR and MU force did not change as a result of the reduction in the number of MUs after N-M suture or by prolonged denervation. However, after prolonged denervation, tetanic and twitch forces both underestimated IR because muscle fiber CSA was lower than normal. This is seen as a shift of the regression lines to higher values of IR (Fig. 7*E,F*).

Discussion

There are two main findings in this study. (1) Prolonged denervation followed by N-N suture results in a reduction in muscle force due primarily to fewer regenerating axons and, to a lesser extent, a reduction in the size of reinnervated muscle fibers. (2) N-M suture, which forces axons to regenerate outside the nerve sheath, simulates the effects of prolonged denervation in reducing the number of regenerating axons and, for long-term denervated muscles, reduces the number of muscle fibers reinnervated by each axon. The present study, therefore, identifies two principal causes for the poor recovery of long-term denervated muscles. First, most axons fail to elongate sufficiently to reach denervated muscle fibers. Second, long-term denervated muscle fibers fail to fully recover from denervation atrophy.

Reduction in the number of reinnervated motor units

For short-term denervation prior to nerve suture, there was no significant change in the number of reinnervated MUs. Thus,

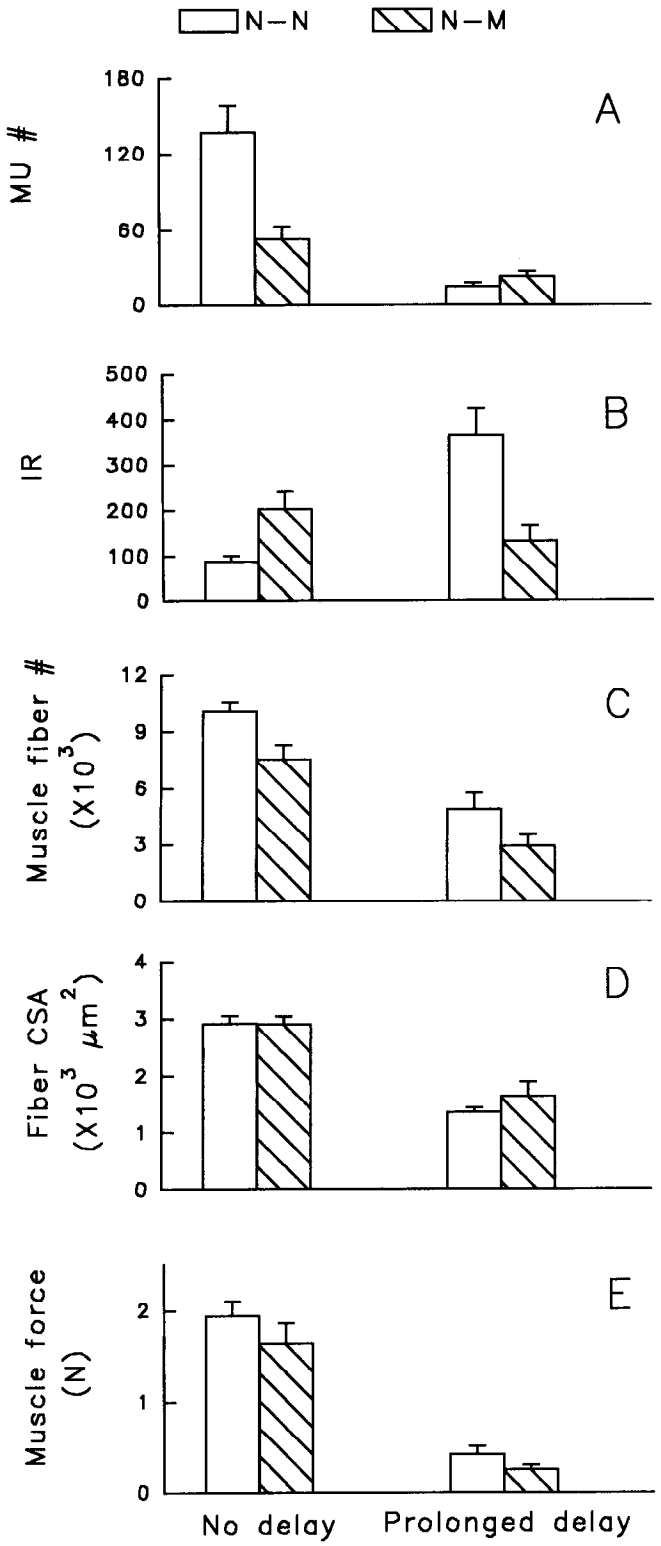

Figure 6. Mean (±SE) MU number (*A*), innervation ratio (IR) (*B*), muscle fiber number (*C*), muscle fiber cross sectional area (*D*), and cross-reinnervated muscle force (*E*) after immediate N-N, N-M sutures, and after prolonged muscle denervation prior to N-N, and N-M suture. Data from reinnervated muscles that were denervated for more than 6 months prior to N-N or N-M suture were pooled for comparison with those after immediate nerve repair.

short-term denervation may not have advantage for promoting muscle reinnervation as previously suggested (Brunetti et al., 1985; Finkelstein et al., 1993). Long-term denervation of the muscle and distal nerve sheaths resulted in drastic reduction in the number of MUs in muscles reinnervated by cross-nerve suture. The fact that poor regeneration after prolonged denervation was simulated by direct nerve–muscle suture strongly suggests that the compromised regeneration is due to progressive deterioration of the intramuscular nerve sheaths, the normal pathways for axonal regeneration (Gutmann and Young, 1944; Sanes et al., 1978; Kuffler, 1986a). Regenerating axons grow in the interface between Schwann cells and their basal laminae, both of which provide substrate and trophic support (Nathaniel and Pease, 1963; Martini and Schachner, 1988; Martini, 1994). There are a number of possible explanations for the progressive deterioration in the ability of intramuscular nerve sheaths to support regenerating axons back to long-term denervated muscle fibers. (1) The initial proliferation of Schwann cells is not maintained and the number of Schwann cells may decrease to a level at which adequate substrate and trophic support is no longer available (Weinberg and Spencer, 1978; Pellegrino and Spencer, 1985; Salonen et al., 1985; Salonen et al., 1987). (2) The basal lamina, which cannot be renewed without Schwann cell–axon contact (Bunge et al., 1982), begins to fragment within weeks of denervation (Giannini and Dyck, 1990). (3) Collagenization of endoneurial tubes may obstruct axonal regeneration (Sunderland and Bradley, 1950a,b). All these factors may contribute to the reduction in the number of axons that eventually reach denervated muscle fibers via the intramuscular nerve sheaths. Morphologically, regenerating axons escape from the deteriorating intramuscular nerve sheaths and grow directly on the surface of denervated muscle fibers (Gutmann and Young, 1944). Such an observation is strongly supported by the present physiological finding that the effect of long-term denervation in reducing MU number was simulated by excluding regenerating axons from the intramuscular nerve sheaths. We found that far fewer axons made functional connections after immediate N-M suture than after immediate N-N suture (Fig. 5). Thus, the surface of denervated muscle fibers is a poor substrate for nerve growth as compared to the nerve sheath. Evidently, the cell adhesion molecules and extracellular matrix proteins expressed on the surface of denervated muscle fibers (Covault et al., 1987) may not provide as favorable substrate or trophic support for axonal elongation as Schwann cells and their basal laminae. Even in freshly denervated muscles, less than 50% of regenerated axons made nerve–muscle connections after N-M suture as compared to N-N suture (Fig. 5). A delay of 2 weeks prior to N-M suture leads to further reduction in the number of reinnervated MUs, indicating that the growth substrate provided by the denervated muscle rapidly deteriorates. The number of reinnervated MUs fell to about 10% when muscle denervation was prolonged beyond 6 months. Eventually, the number of reinnervated MUs after delayed N-N suture fell to the same level as after N-M suture (Fig. 5), suggesting that deterioration of the intramuscular nerve sheaths progresses more slowly than that of the growth substrate provided by denervated muscle.

Regenerating axons that are forced to grow outside the intramuscular nerve sheaths by N-M suture elongate along the denervated muscle fibers. Nonetheless, regenerating axons may still be strongly attracted by the intramuscular nerve sheaths (Kuffler, 1986b, 1987; Diaz and Pecot-Dechavassine, 1990). Axonal regeneration after N-M suture deteriorated rapidly with pro-

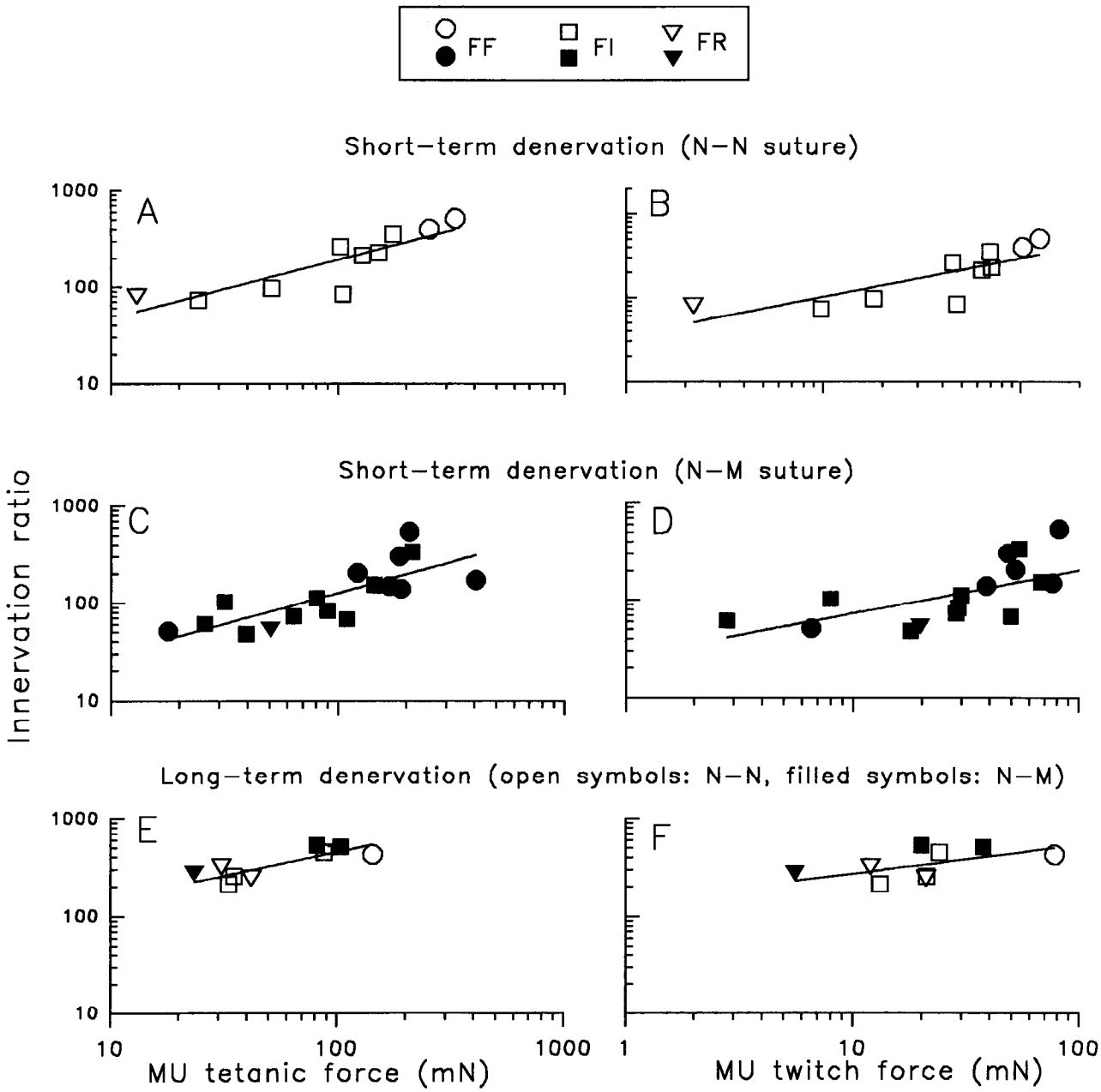

Figure 7. Relationships between MU twitch force, tetanic force, and innervation ratio in cross-reinnervated muscles after short term denervation (<3 months) prior to N-N suture (*A* and *B*), N-M suture (*C* and *D*), and long-term denervation (>6 months) prior nerve repair (*E* and *F*). The slopes between tetanic force and IR, and twitch force and IR were 0.59 ± 0.13 and 0.45 ± 0.13 for short-term denervation prior to N-N suture and were significantly different from zero but not significantly different from each other. The slopes (±SE) of the regression lines are 0.64 ± 0.15 (*C*) and 0.44 ± 0.13 (*D*) for short-term denervation prior to N-M suture that were not different from that for N-N suture. After long-term muscle denervation prior to nerve repair, the regression lines have similar slopes (0.62 ± 0.13 and 0.43 ± 0.17) to those after short denervation prior to nerve repair. However, there were parallel shifts in the regression lines to larger IR values representing the reduced muscle fiber size. MUs were classified physiologically into fast fatiguable (*FF*), fast fatigue intermediate (*FI*), fast fatigue resistant (*FR*).

longed denervation, suggesting that expression of high levels of neural cell adhesion molecules, for example, may not be sustained. The production of extracellular matrix molecules such as fibronectin and heparin-sulphate proteoglycan may fall with time. An alternative is that the Schwann cells that migrate from the endplate region also fail to support regenerating axons. Furthermore, as the intramuscular nerve sheaths deteriorate, they may fail to attract and provide support for regenerating axons. These changes may also explain why IR is significantly less after N-M than N-N suture (Fig. 6).

Reduction in the number of reinnervated muscle fibers

The reduction in the number of reinnervated muscle fibers could result from a reduction in (1) the number of muscle fibers that are viable for reinnervation (Anzil and Wernig, 1989), (2) the ability of long-term denervated muscle fibers to accept reinner-

vation (Gutmann and Young, 1944), and/or (3) the number of axons that succeed in elongating to reach the denervated muscle fibers and making functional connections.

Although long-term denervated muscle fibers undergo extensive degeneration and atrophy (Gutmann and Young, 1944; Anzil and Wernig, 1989; Schmalbruch et al., 1991), regeneration of new fibers from satellite cells is often sufficient to maintain the normal number of viable muscle fibers even 10 months after denervation (Mussinin et al., 1987; Schmalbruch et al., 1991). Unless the satellite cell pool is exhausted, a reduction in the number of viable muscle fibers cannot account for the ultimate reduction in the number of reinnervated muscle fibers after delayed nerve repair (Gutmann and Zelena, 1962; Anzil and Wernig, 1989). The postulate of a lack of viable muscle fibers is further challenged by the finding that the threefold increase in the number of muscle fibers reinnervated by each axon is the same as after immediate nerve repair (Fu et al., 1991; Rafuse, 1993) and after partial denervation (Rafuse et al., 1992; Gordon et al., 1993). Since each motor axon supplies as many muscle fibers as possible, poor reinnervation is likely because few axons have succeeded in regenerating and reinnervating denervated muscle fibers. Even in short-term denervated muscles where degeneration is minimal, we observed the same small number of reinnervated MUs after N-M suture that was associated with a significant reduction in the number of regenerating axons. Thus, poor reinnervation of long-term denervated muscle is far more likely due to failure of many regenerating axons to elongate and/or make synaptic connections with denervated muscle fibers than a reduction in the number of viable muscle fibers.

The present finding that the enlarged MUs in a reinnervated muscle contained an increasing number of muscle fibers also argues against the idea that long-term denervated muscle fibers fail to accept reinnervation (see Gutmann and Young, 1944). It is more likely that not all muscle fibers are reinnervated because each of the few regenerated motor axons cannot reinnervate more than five times the normal number of muscle fibers. This is in agreement with previous studies showing that after prolonged denervation the number of reinnervated muscle fibers is reduced when fewer axons have regenerated (Anzil and Wernig, 1989) and remains the same as normal when the number of successfully regenerated axons is maximized by repeated freezing of the sciatic nerve (Irintchev et al., 1990).

Incomplete recovery of muscle fiber size from denervation atrophy

Failure of reinnervated muscle fibers to resume their normal size suggests that atrophy can only be partially reversed after prolonged denervation, possibly due to exhaustion of satellite cells. After prolonged denervation, viable muscle fibers are very small (Gutmann, 1948; Sunderland and Ray, 1950; Schmalbruch et al., 1991). Reversal of denervation atrophy, possibly as in postnatal enlargement of muscle fiber diameter, may rely on incorporation of satellite cells as a source of new nuclei (Moss and LeBlond, 1971; Mazanet and Franzini-Armstrong, 1986). However, the satellite cell pool is likely depleted in prolonged denervation. We are currently testing this possibility by irradiation of denervated muscle after reinnervation. An additional factor that may limit muscle recovery from denervation atrophy is endomysial fibrosis and connective tissue accumulation (Gutmann and Young, 1944; Savolainen et al., 1988). Both may physically limit the size of muscle fibers.

Conclusions

The number of regenerating motor axons that reinnervate denervated muscle fibers is profoundly reduced when denervation is prolonged as a result of deterioration of the intramuscular nerve sheaths. In addition, long-term denervated muscle fibers fail to fully recover from denervation atrophy, possibly due to exhaustion of the satellite cell population. Thus, prolonged denervation after delayed nerve repair is very detrimental to functional recovery and accounts for a 90% reduction in the number of functional MUs. This compares with a 30% reduction in prolonged axotomy (Fu and Gordon, 1995). These findings provide a basis for surgical repair of nerve injuries. Efforts should be devoted to optimizing nerve regeneration in the intramuscular nerve sheaths and denervated muscles in addition to minimizing the effects of denervation atrophy.

References

Anzil AP, Wernig A (1989) Muscle fibre loss and reinnervation after long-term denervation. J Neurocytol 18:833–845.

Brunetti O, Carretta M, Magni F, Pazzaglia U (1985) Role of the interval between axotomy and nerve suture on the success of muscle reinnervation: an experimental study in the rabbit. Exp Neurol 90: 308–321.

Bunge MB, Williams AK, Wood PM (1982) Neuron–Schwann cell interaction in basal lamina formation. Dev Biol 92:449–460.

Covault J, Cunningham JM, Sanes J (1987) Neurite outgrowth on cryostat sections of innervated and denervated skeletal muscle. J Cell Biol 105:2479–2488.

Diaz J, Pecot-Dechavassine M (1990) Nerve sprouting induced by a piece of peripheral nerve placed over a normally innervated frog muscle. J Physiol (Lond) 421:123–133.

Finkelstein DI, Dooley PC, Luff AR (1993) Recovery of muscle after different periods of denervation and treatments. Muscle Nerve 16: 769–777.

Fu SY, Gordon T (1993a) A quantitative study of the basis for the poor functional recovery after delayed nerve repair in rats. 5th Int Symp Neural Regen 28:45.

Fu SY, Gordon T (1993b) Prolonged denervation is a major contributing factor to poor motor recovery after delayed peripheral nerve repair. Physiol Can 24:137.

Fu SY, Gordon T (1995) Contributing factors to poor functional recovery after delayed nerve repair: prolonged axotomy. J Neurosci 15: 3876–3885.

Fu S, Tyreman N, Gordon T (1991) Reinnervation of rat tibialis anterior muscles by tibial nerve after prolonged denervation or axotomy. Soc Neurosci Abstr 17:937.

Fu SY, Gordon T, Parry DJ, Tyreman N (1992) Immunohistochemical analysis of fiber types within physiologically typed motor units of rat tibialis anterior muscle after long-term cross-reinnervation. Soc Neurosci Abstr 18:1557.

Fu SY, Raji M, Tyreman N, Gordon T (1993) Does long term axotomy compromise nerve regeneration. Soc Neurosci Abstr 19:423.

Giannini C, Dyck PJ (1990) The fate of Schwann cell basement membranes in permanently transected nerves. J Neuropathol Exp Neurol 49:550–563.

Gordon T, Yang JF, Stein RB, Tyreman N (1993) Recovery potential of the neuromuscular system after nerve injuries. Brain Res Bull 30: 477–482.

Gutmann E (1948) Effect of delay of innervation on recovery of muscle after nerve lesions. J Neurophysiol 11:279–294.

Gutmann E, Young JZ (1944) The re-innervation of muscle after various periods of atrophy. J Anat 78:15–44.

Gutmann E, Zelena J (1962) Morphological changes in the denervated muscle. In: The denervated muscle (Gutmann E, ed), pp 57–102. Prague: Czechoslovak Academy of Sciences.

Holmes W, Young JZ (1942) Nerve regeneration after immediate and delayed suture. J Anat 77:63–108.

Irintchev A, Draguhn A, Wernig A (1990) Reinnervation and recovery of mouse soleus muscle after long-term denervation. Neuroscience 39:231–243.

Jansen JKS, Fladby T (1990) The perinatal reorganization of the innervation of skeletal muscle in mammals. Prog Neurobiol 34:39–90.
Kuffler DP (1986a) Accurate reinnervation of motor end plates after disruption of sheath cells and muscle fibres. J Comp Neurol 250: 228–235.
Kuffler DP (1986b) Isolated satellite cells of a peripheral nerve direct the growth of regenerating frog axons. J Comp Neurol 249:57–64.
Kuffler DP (1987) Long-distance regulation of regenerating frog axons. J Exp Biol 132:151–160.
Martini R (1994) Expression and functional roles of neural cell surface molecules and extracellular matrix components during development and regeneration of peripheral nerves. J Neurocytol 23:1–28.
Martini R, Schachner M (1988) Immunoelectron microscopic localization of neural cell adhesion molecules (L1, N-CAM, and myelin-associated glycoprotein) in regenerating adult mouse sciatic nerve. J Cell Biol 106:1735–1746.
Mazanet R, Frazini-Armstrong C (1986) The satellite cell. In: Basic and clinical myology, Vol I (Engel AG, Banker BQ, eds), pp 285–307. New York: McGraw-Hill.
McComas AJ (1991) Invited review: motor unit estimation: methods, results, and present status. Muscle Nerve 14:585–597.
Moss FP, Leblond CP (1971) Satellite cells as a source of nuclei in muscles of growing rats. Anat Rec 170:421–436.
Mussini I, Favaro G, Carraro U (1987) Maturation, dystrophic changes and the continuous production of fibres in skeletal muscle regenerating in the absence of nerve. J Neuropathol Exp Neurol 46:315–331.
Nathaniel EJ, Pease DC (1963) Regenerative changes in rat dorsal roots following Wallerian degeneration. J Ultrastruct Res 9:533–549.
Parry DJ, Wilkinson RS (1990) The effect of reinnervation on the distribution of muscle fibre types in the tibialis anterior muscle of the mouse. Can J Physiol Pharmacol 68:596–602.
Pellegrino RG, Spencer PS (1985) Schwann cell mitosis in response to regenerating peripheral axons *in vivo*. Brain Res 342:16–25.
Pullen AH (1977) The distribution and relative sizes of three histochemical fibre types in the rat tibialis anterior muscle. J Anat 123:1–19.
Rafuse VF (1993) Motor unit size after nerve injuries. Ph.D thesis, University of Alberta.
Rafuse VF, Gordon T, Orozco R (1992) Proportional enlargement of motor units after partial denervation of cat triceps surae muscles. J Neurophysiol 68:1261–1276.
Salonen V, Lehto M, Kalimo H, Penttinen R, Aho H (1985) Changes in intramuscular collagen and fibronectin in denervation atrophy. Muscle Nerve 8:124–131.
Salonen V, Peltonen J, Roytta M, Virtanen I (1987) Laminin in traumatised peripheral nerve: basement membrane changes during degeneration and regeneration. J Neurocytol 16:713–720.
Sanes JR, Marshall LM, McMahan UJ (1978) Reinnervation of muscle fibre basal lamina after removal of myofibres. Differentiation of regenerating axons at original synaptic sites. J Cell Biol 78:667–678.
Savolainen J, Myllyla V, Myllyla R, Vihko V, Vaananen K, Takala TES (1988) Effects of denervation and immobilization on collagen synthesis in rat skeletal muscle and tendon. Am J Physiol 254:R897–R902.
Schmalbruch H, Al-Amood WS, Lewis DM (1991) Morphology of long-term denervated rat soleus muscle and the effect of chronic electrical stimulation. J Physiol (Lond) 441:233–241.
Stein RB, Yang JF (1990) Methods for estimating the number of motor units in human muscles. Ann Neurol 28:487–495.
Sunderland S (1978) Nerve and nerve injuries. Edinburgh: Livingstone.
Sunderland S, Bradley KC (1950a) Denervation atrophy of the distal stump of a severed nerve. J Comp Neurol 93:401–409.
Sunderland S, Bradley KC (1950b) Endoneurial tube shrinkage in the distal segment of a severed nerve. J Comp Neurol 93:411–420.
Sunderland S, Ray LJ (1950) Denervation changes in mammalian striated muscle. J Neurol Neurosurg Psychiatry 13:159–177.
Terzis JK, Smith KL (1990) The peripheral nerve: structure, function and reconstruction. New York: Raven.
Tötösy de Zepetnek JE, Zung HV, Erdebil S, Gordon T (1992a) Innervation ratio is an important determinant of force in normal and reinnervated rat tibialis anterior muscles. J Neurophysiol 67:1385–1403.
Tötösy de Zepetnek JE, Zung HV, Erdebil S, Gordon T (1992b) Motor unit categorization on the basis of contractile and histochemical properties: a glycogen depletion analysis of normal and reinnervated rat tibialis anterior muscles. J Neurophysiol 67:1404–1415.
Weinberg HJ, Spencer PS (1978) The fate of Schwann cells isolated from axonal contact. J Neurocytol 7:555–569.